TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35.U.S.C. 371

EXPRESS MAIL LABEL No	DATE
EK839858763US	August 16, 2000

ATTORNEY'S DOCKET NO
A33251

US APPLICATION NO
To Be Assigned 09/622385

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP99/01017	February 18, 1999	February 2, 1998

TITLE OF INVENTION
Process for preparing trifluoro-3(R)-hydroxybutyric acid derivatives

APPLICANT(S) FOR DO/EO/US
Lonza, AG (Geschaftsleitung: 4002 Basel), CH-3945 Gampel CH

Applicant herewith submits to the United States Designated /Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(I).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [X] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [X] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [X] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Deposit Receipts of Microorganisms DSM 12566 and DSM 11902.

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533 Rec'd PCT/PTO 16 AUG 2000

17. [X] The following fees are submitted: CALCULATIONS PTO USE ONLY

Basic National Fee (37 CFR 1.492(a)(1)-(5):

Neither international preliminary examination fee (37 CFR 1.482)

Nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO (1.492(a)(3)) $970.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO (1.492(a)(5) $840.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO(1.492(a)(2)) $690.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) (1.492(a)(1)) $670.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... $ 96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =	$840.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] **20** [X] **30** months from the earliest claimed priority date (37 C.F.R. 1.492(e)).	$130.00	

Claims	**Number Filed**	**Number Extra**	**Rate**	$	
Total Claims	6-20=		X $ 18.00	$	
Independent Claims	1-3=		X $ 78.00	$	
Multiple dependent claim(s) (if applicable)			+ $260.00	$	
TOTAL OF ABOVE CALCULATIONS =				$970.00	
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$970.00	
Processing fee of $130.00 for furnishing the English translation later than [ ] **20** [ ] **30** months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$970.00	
				Amt. refunded	$
				charged	$970.00

a. [X] A check in the amount of $970.00 to cover the above fees is enclosed.

b. [ ] Please charge our Deposit Account No. 02-4377 in amount of $___ to cover the above fees. A copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4377. A copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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Aug. 16, 2000

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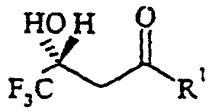
Process for preparing trifluoro-3(R)-hydroxybutyric acid derivatives

The invention relates to a novel biotechnological process for preparing trifluoro-3(R)-hydroxybutyric acid derivatives of the general formula



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Trifluoro-3(R)-hydroxybutyric acid derivatives such as ethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate are important intermediates for preparing pharmaceuticals, for example for preparing Befloxatone, a monoamine oxidase A inhibitor (EP-A-0 736 606).

Several biotechnological processes for preparing 4,4,4-trifluoro-3(R)-hydroxybutyric esters have already been disclosed.

Guerrero, A. & Raja, E. (Bioorganic Chemistry Letters 1(12), 675-678) describe a microbiological process for preparing ethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate using Saccharomyces cerevisae and proceeding from the corresponding racemate. In this method, the enantiomeric purity of the resulting desired product is poor.

EP-A-0 736 606 describes a biotechnological process for preparing ethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate which uses the lipase Novozym 435 and which proceeds from ethyl 4,4,4-trifluoro-3-hydroxybutyrate. The disadvantage of this process is the indifferent yield of the desired product.

EP-A-0 577 446 includes a biotechnological process for preparing optically active ethyl 4,4,4-trifluoro-3-hydroxybutyrate which uses lipases and proceeds from the corresponding racemic ester. When this process is used, the product is obtained in low yield and its optical purity is poor.

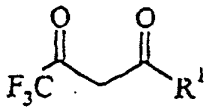
WO 89/02 470 describes a process for preparing ethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate which uses hydrolytic enzymes and which proceeds from racemic

ethyl 4,4,4-trifluoro-3-acyloxybutyrate. However, this process does not yield the corresponding product in enantiomerically pure form.

The object of the present invention was to make available a biotechnological process for preparing 4,4,4-trifluoro-3(R)-hydroxybutyric acid derivatives which enables the desired product to be isolated in good yield and at a good level of optical purity.

This object is achieved using the process according to Claim 1.

According to the invention, the process is carried out by a trifluoroacetoacetic acid derivative of the general formula



II

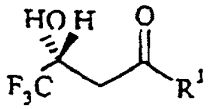
in which

R^1 is $-OR^2$, in which R^2 is hydrogen, C_{1-10}-alkyl, C_{1-10}-alkenyl, C_{3-8}-cycloalkyl, aryl, alkoxyalkyl or alkoxyalkoxyalkyl,

$-NR^3R^4$, in which R^3 and R^4 are identical or different and represent hydrogen, C_{1-10}-alkyl, C_{1-10}-alkenyl, C_{3-8}-cycloalkyl or aryl, or

$-SR^5$, in which R^5 is hydrogen, C_{1-10}-alkyl, C_{1-10}-alkenyl, aryl or C_{3-8}-cycloalkyl,

being converted by means of microorganisms which are able to reduce a carbonyl function, or by means of a cell-free enzyme extract of these microorganisms, into the compound of the general formula



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in which R^1 has the said meaning.

In that which follows, a branched or unbranched, primary, secondary or tertiary aliphatic group, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, isopentyl, sec-pentyl, hexyl, heptyl, octyl, nonyl or decyl can be

used as C_{1-10}-alkyl. C_{1-10}-alkyl preferably denotes ethyl, propyl, isopropyl or hexyl.

Ethenyl, propenyl, allyl and butenyl can, for example, be used as C_{1-10}-alkenyl. Allyl is preferably used.

Aryl preferably denotes substituted or unsubstituted benzyl, phenyl or naphthyl. Halogenated benzyl, such as chloro- or bromobenzyl, can, for example, be used as substituted benzyl. Unsubstituted benzyl is preferably employed.

C_{3-8}-cycloalkyl preferably denotes cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl or cyclooctyl, preferably cyclohexyl.

Alkoxyalkyl preferably denotes C_{1-6}-alkoxyethyl such as methoxyethyl and ethoxyethyl, particularly preferably ethoxyethyl.

Alkoxyalkoxyalkyl preferably denotes 2-(2-C_{1-6}-alkoxy-ethoxy)ethyl, such as 2-(2-methoxy-ethoxy)ethyl and 2-(2-ethoxyethoxy)ethyl, with the latter being particularly preferably employed.

Consequently, preferred starting compounds are ethyl trifluoroacetoacetate, propyl trifluoroaceto-acetate, isopropyl trifluoroacetoacetate and hexyl trifluoroacetoacetate, cyclohexyl trifluoroaceto-acetate, benzyl trifluoroacetoacetate, ethoxyethyl trifluoroacetoacetate and ethoxyethoxyethyl trifluoro-acetoacetate.

Examples of expedient microorganisms which are able to reduce a carbonyl function are microorganisms which contain an expressable gene for an enzyme which is able to reduce a carbonyl function, for example an enzyme possessing reductase activity, in particular a gene for an aldehyde reductase, an alcohol dehydrogenase or a ketone reductase. The enzymes which are able to reduce a carbonyl function can be NADPH (β-nicotinamide adenine dinucleotide phosphate)-dependent or be dependent on other cofactors. Preference is given to using microorganisms which contain NADPH-dependent reduction systems.

Cell-free enzyme extracts of these microorganisms can be obtained by means of methods which are customary to the skilled person, for example by means of the French press method, the ultrasonication method or the lysozyme method.

The biotransformation is expediently carried out using microrganisms which contain an aldehyde reductase, in particular an NADPH-dependent aldehyde reductase.

Microorganisms which contain an NADPH-dependent aldehyde reductase, such as microorganisms of the species Sporobolomyces salmonicolor, have already been described by Shimizu et al., 1990, Applied and Environmental Microbiology, 56(8), 2374-2377 and Kataoka, M. et al., Biochimica et Biophysica Acta, 1112, 57-62 (1992). These microorganisms can, on the one hand, be used themselves for the process according to the invention and, on the other hand, serve as the starting material for constructing plasmids and other suitable microorganisms.

Recombinant microorganisms which are transformed with a gene encoding an enzyme which is able to reduce a carbonyl function are expediently employed for the biotransformation. Examples of microorganisms which can be transformed with such a gene are microorganisms of the genus Escherichia, in particular the species Escherichia coli, for example Escherichia coli JM109, Escherichia coli DH5 and Escherichia coli HB101.

The gene possessing the reductase activity, for example an aldehyde reductase, is preferably located on a vector which is suitable for the transformation, for example a plasmid, expediently together with a promoter which is suitable for expressing the gene, such as the tac promoter (P_{tac}).

Provided the microorganisms employed contain NADPH-dependent enzymes, the biotransformation is expediently carried out in the presence of NADPH. The NADPH is either added directly in the requisite

quantities or produced in situ. Advantageously, the NADPH is produced in situ. For this purpose, the biotransformation is expediently carried out in the presence of an NADPH generator or regenerator, i.e. an enzyme which catalyzes the formation of NADPH from its oxidized form, i.e. $NADP^+$. A glucose dehydrogenase, for example Bacillus megaterium glucose dehydrogenase, is expediently employed as the NADPH generator or regenerator.

In order to generate NADPH during the biotransformation, the latter is expediently carried out in the presence of a microorganism which expresses the NADPH generator. Recombinant microorganisms which are transformed with the gene encoding the NADPH generator are, in particular, used for this purpose. In this case, the gene for the NADPH generator is located on a vector which is suitable for the transformation, for example a plasmid, expediently together with a promoter which is suitable for expressing the gene, such as the tac promoter (P_{tac}).

Different microorganisms, one of which is able to reduce the carbonyl function and one of which is able to form NADPH, can be employed for preparing the trifluoro-3(R)-hydroxybutyric acid derivatives of the general formula I using, in the presence of an NADPH generator, a microorganism which contains an NADPH-dependent enzyme which is capable of reducing a carbonyl function, for example an NADPH-dependent aldehyde reductase. However, the microorganisms which are used in accordance with the invention, and which are able to reduce a carbonyl function, advantageously already themselves contain a gene which encodes an NADPH generator or regenerator, for example a gene which encodes a glucose dehydrogenase.

Recombinant microorganisms which are transformed with a gene which encodes an NADPH-dependent enzyme, for example a gene which encodes an NADPH-dependent aldehyde reductase, and also a gene which encodes an NADPH generator or regenerator, for

example a gene which encodes a glucose dehydrogenase, are advantageously employed for the biotransformation. In one possible embodiment, these genes are located for expression on one single plasmid. In another embodiment, these genes are present on different, mutually compatible plasmids.

Consequently, the biotransformation can advantageously be carried out using microorganisms which contain:

- at least one vector, for example a plasmid, which contains a gene for an enzyme which is capable of reducing a carbonyl function, for example an aldehyde reductase gene;
- at least two vectors, for example plasmids, one of which contains a gene for an enzyme capable of reducing a carbonyl function, for example an aldehyde reductase gene, while the other contains a gene for an NADPH generator or regenerator, for example a glucose dehydrogenase gene; or
- at least one vector, for example a plasmid, which contains both a gene for an enzyme which is capable of reducing a carbonyl function, for example an aldehyde reductase gene, and also a gene for an NADPH generator or regenerator, for example a glucose dehydrogenase gene.

Advantageously, the biotransformation is carried out using microorganisms of the species E. coli JM109 or E. coli DH5 which are transformed with at least two plasmids which respectively contain an aldehyde reductase gene and a glucose dehydrogenase gene, or using microorganisms of the species E. coli HB101 or E. coli DH5 which are transformed with at least one plasmid which contains both genes, i.e. the aldehyde reductase gene and the glucose dehydrogenase gene. In particular, the biotransformation is carried out using E. coli JM109 and E. coli DH5 which contain an aldehyde reductase gene and a glucose dehydrogenase gene. Naturally, the biotransformation can also be

carried out using different microorganisms which in each case contain only one of the said genes.

Fig. 1 shows the structure of a plasmid, pKAR, which is suitable for the present invention and which contains the gene for the Sporobolomyces salmonicolor NADPH-dependent aldehyde reductase together with the P_{tac} promoter and an ampicillin (Ap) resistance as the selection marker.

Fig. 2 shows the structure of another plasmid, pKKGDH, which is suitable for the present invention and which contains the gene for the Bacillus megaterium glucose dehydrogenase together with the P_{tac} promoter and a kanamycin (Km) resistance as the selection marker.

The microorganism E. coli JM109, harbouring the plasmid pKAR, containing a gene encoding the Sporobolomyces salmonicolor NADPH-dependent aldehyde reductase, and the plasmid pKKGDH, containing a gene encoding the Bacillus megaterium glucose dehydrogenase, was deposited in the Deutsche Sammlung von Mikroorganismen and Zellkulturen [German Collection of Microorganisms and Cell Cultures] GmbH (DSMZ), D-38124 Braunschweig, Mascheroderweg 1b, Germany, under designation DSM 11902, in accordance with the Budapest Treaty, on 16.12.1997. The microorganism E. coli DH5, harbouring the plasmids pKAR and pKKGDH, was deposited in the abovementioned depository institution under designation DSM 12566, in accordance with the Budapest Treaty, on 7.12.1998.

The genes can be expressed in dependence on the expression system. In the case of the expression systems which are preferably used in accordance with the invention, the expression of the genes can, for example, be induced with IPTG (isopropylthio-galactoside) if E. coli JM109 or E. coli HB101 is used as the microorganism. As the skilled person knows, induction with IPTG is not necessary when E. coli DH5 is used.

Following customary culture of the cells, the biotransformation can be carried out in a single-phase or two-phase system, preferably in a two-phase system.

Buffer media which are customary to the skilled person, such as low molecular weight phosphate buffers or Tris buffers, can be employed as a single-phase system.

The said buffer media which are customary to the skilled person, together with an organic solvent in which the starting compound is soluble, can be used as a two-phase system. Examples of suitable organic solvents are esters, alcohols, halogenated hydrocarbons, ethers, aliphatic C_{5-12}-hydrocarbons or aromatic hydrocarbons. Acetic esters, such as methyl acetate, ethyl acetate, propyl acetate and butyl acetate, can be used as esters. C_{4-10}-alcohols, such as hexanol, heptanol and octanol, can be used as alcohols. Benzene, toluene and xylene can, for example, be used a aromatic hydrocarbons. Chloroform and dichloromethane can, for example, be used as halogenated hydrocarbons. Diethyl ether, tetrahydrofuran, methyl tert-butyl ether and dibutyl ether can, for example, be used as ethers. Examples of suitable aliphatic C_{5-12}-hydrocarbons are pentane, hexane, heptane, octane, nonane and decane.

A two-phase system in which the second phase consists of the starting compound and/or product is also suitable. Cosolvents can be employed for increasing the solubility of the starting compound. Either low molecular weight aliphatic alcohols, such as methanol, ethanol, propanol, isopropanol or tert-butanol, or inert solvents, such as dimethyl sulphoxide, acetone and acetonitrile, can be used as cosolvents.

The biotransformation is customarily carried out in the presence of a C source. Examples of suitable C sources are carbohydrates such as glucose, fructose or sucrose, and sugar alcohols, such as glycerol.

The pH of the media can be in a range of from 5 to 10, preferably of from 6 to 8.

The biotransformation is expediently carried out at a temperature of from 5 to 60°C, preferably of from 10 to 40°C.

After a reaction time of from a few minutes to 50 h, the desired product can then be isolated in high yield and at high enantiomeric purity (ee).

Examples

Example 1

Culturing the microorganisms

E. coli JM109/pKAR,pKKGDH (DSMZ 11902) cells were cultured at 22°C in 12 l of mineral salt medium (Table 1) in a 20 l fermenter. After 6 h, IPTG was added in order to induce the cells. Glycerol was then added and the cells were cultured, within 52 h, up to an optical density of OD_{650nm} = 41.8. The cells were then stored at -80°C.

Table 1

Yeast extract	0.5	g/l
Glycerol	30	g/l
$MgCl_2 \times 6H_2O$	0.8	g/l
$CaCl_2$	0.16	g/l
$(NH_4)_2 SO_4$	2.0	g/l
SLF solution	1.0	ml/l
Fe-EDTA solution	1.5	ml/l
PPG-2000	0.1	g/l
$Na_2HPO_4 \times 2H_2O$	1.0	g/l
KH_2PO_4	1.0	g/l
K_2HPO_4	1.0	g/l
Thiamine	10	mg/l

SLF solution:

KOH	15.1	g/l
$?H_2O$	100	g/l
		g/l
MnC_		
H_3BO_3	2.7	g/l
$CoCl_3 \times 6H_2O$	1.8	g/l
$CuCl_2 \times 2H_2O$	1.5	g/l
$NiCl_2 \times 6H_2O$	0.18	g/l
$Na_2MoO_4 \times 2H_2O$	0.27	g/l

Fe-EDTA solution:

KOH	10	g/l
$EDTANa_2 \times 2H_2O$	50	g/l
$FeSO_4 \times 7H_2O$	20	g/l

Example 2

Preparation of ethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate

a) 140 g of glucose and 0.56 g of $NADP^+$ were added to 800 ml of mineral salt medium (Table 1) containing E. coli JM109/pKAR,pKKGDH at an OD_{650nm} of 7.2. 400 ml of butyl acetate containing 70 g of ethyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was placed in a 2 l fermenter, stirred at 400 rpm and gassed with air (400 ml/min). The pH was kept at 6.0 by adding 1 M Na_2CO_3. After 24 h, the organic phase contained 48 g of ethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an ee value of >99%, corresponding to a molar yield of 67.8%.

b) 140 g of glucose and 0.56 g of $NADP^+$ were added to 800 ml of potassium phosphate buffer (100 mM, pH 6.0) containing the microorganisms according to Example 1 at an OD_{650nm} of 30.7. 400 ml of butyl acetate containing 70 g of ethyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was fed into a fermenter as described in Example 2a. The pH was kept at pH 6.0 by adding 1 M Na_2CO_3. After 25 h, a further 10 g of ethyl 4,4,4-trifluoroacetoacetate were added. After 45 h, the organic phase contained 49 g of ethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an ee value of >99%, corresponding to a molar yield of 60.6%.

c) 140 g of glucose and 50 mg of $NAPD^+$ were added to 800 ml of potassium phosphate buffer (0.1 M, pH 6.0) containing E. coli JM109/pKAR,pKKGDH at an OD_{650nm} of 7.6. 400 ml of butyl acetate containing 70 g of ethyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was placed in a 2 l fermenter, stirred at 400 rpm and gassed with air (400 ml/min). The pH was kept at 6.0 by adding 1 M Na_2CO_3. A further 50 mg of $NADP^+$ were added 5 h after starting the fermenter. After 24 h, the organic phase contained 50 g of ethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an

ee value of >99.8%, corresponding to a molar yield of 71%.

d) 140 g of glucose and 50 mg of $NAPD^+$ were added to 800 ml of potassium phosphate buffer (0.1 M, pH 6.0) containing E. coli DH5/pKAR,pKKGDH at an OD_{650nm} of 6.5. 400 ml of butyl acetate containing 70 g of ethyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was placed in a 2 l fermenter, stirred at 400 rpm and gassed with air (400 ml/min). The pH was kept at 6.0 by adding 1 M Na_2CO_3. A further 50 mg of $NADP^+$ were in each case added after 5 h and after 26 h. After 46 h, the organic phase contained 35 g of ethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an ee value of >99.7%, corresponding to a molar yield of 51%.

Example 3

Preparation of isopropyl 4,4,4-trifluoro-3(R)-hydroxybutyrate

a) 140 g of glucose and 0.56 g of $NADP^+$ were added to 800 ml of mineral salt medium in accordance with Example 1 containing E. coli JM109/pKAR,pKKGDH at an OD_{650nm} of 9.7. 400 ml of butyl acetate containing 70 g of isopropyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was fed into fermenter as described in Example 2. The pH was kept at pH 6.0 by adding 1 M Na_2CO_3. After 21 h, the organic phase contained 42.2 g of isopropyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an ee value of >99%, corresponding to a molar yield of 59.7%.

b) 140 g of glucose and 50 mg of $NADP^+$ were added to 800 ml of potassium phosphate buffer (0.1 M, pH 6.0) containing E. coli DH5/pKAR,pKKGDH at an OD_{650nm} of 8.5. 400 ml of butyl acetate containing 70 g of isopropyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was placed in a 2 l fermenter, stirred at 400 rpm and gassed with air (400 ml/min). The pH was kept at 6.0 by adding 1 M Na_2CO_3. A further 50 mg of $NADP^+$ were added 5 h after starting the

fermenter. After 24 h, the organic phase contained 32 g of isopropyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an ee value of >99.9%, corresponding to a molar yield of 46%.

Example 4

Preparation of hexyl 4,4,4-trifluoro-3(R)-hydroxybutyrate

140 g of glucose and 50 mg of $NADP^+$ were added to 800 ml of potassium phosphate buffer (0.1 M, pH 6.0) containing E. coli DH5/pKAR,pKKGDH at an OD_{650nm} of 9.5. 400 ml of butyl acetate containing 70 g of hexyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was placed in a 2 l fermenter, stirred at 400 rpm and gassed with air (400 ml/min). The pH was kept at pH 6.0 by adding 1 M Na_2CO_3. A further 50 mg of $NAPD^+$ were added 5 h after starting the fermenter. After 24 h, the organic phase contained 2 g of hexyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an ee value of >99.9%, corresponding to a molar yield of 3%.

Example 5

Preparation of cyclohexyl 4,4,4-trifluoro-3(R)-hydroxybutyrate

140 g of glucose and 50 mg of $NADP^+$ were added to 800 ml of potassium phosphate buffer (0.1 M, pH 6.0) containing E. coli DH5/pKAR,pKKGDH at an OD_{650nm} of 8.9. 400 ml of butyl acetate containing 70 g of cyclohexyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was placed in a 2 l fermenter, stirred at 400 rpm and gassed with air (400 ml/min). The pH was kept at pH 6.0 by adding 1 M Na_2CO_3. A further 50 mg of $NAPD^+$ were added 5 h after starting the fermenter. After 24 h, the organic phase contained 16 g of cyclohexyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an ee value of >99.9%, corresponding to a molar yield of 23%.

Example 6

Preparation of benzyl 4,4,4-trifluoro-3(R)-hydroxybutyrate

140 g of glucose and 50 mg of $NADP^+$ were added to 800 ml of potassium phosphate buffer (0.1 M, pH 6.0) containing E. coli DH5/pKAR,pKKGDH at an OD_{650nm} of 9.0. 400 ml of butyl acetate containing 70 g of benzyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was placed in a 2 l fermenter, stirred at 400 rpm and gassed with air (400 ml/min). The pH was kept at pH 6.0 by adding 1 M Na_2CO_3. A further 50 mg of $NAPD^+$ were added 5 h after starting the fermenter. After 24 h, the organic phase contained 6 g of benzyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an ee value of >99.9%, corresponding to a molar yield of 9%.

Example 7

Preparation of 2-ethoxyethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate

105 g of glucose and 37.5 mg of $NADP^+$ were added to 600 ml of potassium phosphate buffer (0.1 M, pH 6.0) containing E. coli DH5/pKAR,pKKGDH at an OD_{650nm} of 10.2. 300 ml of butyl acetate containing 35 g of ethoxyethyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was placed in a 2 l fermenter, stirred at 400 rpm and gassed with air (300 ml/min). The pH was kept at pH 6.0 by adding 1 M Na_2CO_3. A further 37.5 mg of $NAPD^+$ were added 5 h after starting the fermenter. After 24 h, the organic phase contained 4 g of ethoxyethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an ee value of 98.6%, corresponding to a molar yield of 12%.

Example 8

Preparation of 2-(2-ethoxyethoxy)ethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate

105 g of glucose and 37.5 mg of $NADP^+$ were added to 600 ml of potassium phosphate buffer (0.1 M, pH 6.0) containing E. coli DH5/pKAR,pKKGDH at an OD_{650nm} of 10.7. 300 ml of butyl acetate containing 35 g of ethoxyethoxyethyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was placed in a 2 l fermenter, stirred at 400 rpm and gassed with air (300 ml/min). The pH was kept at pH 6.0 by adding 1 M Na_2CO_3. A further 37.5 mg of $NAPD^+$ were added 5 h after starting the fermenter. After 24 h, the organic phase contained 5 g of ethoxyethoxyethyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an ee value of >99.9%, corresponding to a molar yield of 16%.

Example 9

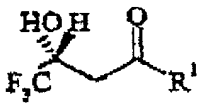
Preparation of methyl 4,4,4-trifluoro-3(R)-hydroxybutyrate

105 g of glucose and 37.5 mg of $NADP^+$ were added to 600 ml of potassium phosphate buffer (0.1 M, pH 6.0) containing E. coli DH5/pKAR,pKKGDH at an OD_{650nm} of 11.4. 300 ml of butyl acetate containing 33 g of methyl 4,4,4-trifluoroacetoacetate were added and the resulting mixture was placed in a 2 l fermenter, stirred at 400 rpm and gassed with air (300 ml/min). The pH was kept at pH 6.0 by adding 1 M Na_2CO_3. A further 37.5 mg of $NAPD^+$ were added 5 h after starting the fermenter. After 24 h, the organic phase contained 3.6 g of methyl 4,4,4-trifluoro-3(R)-hydroxybutyrate having an ee value of 96.1%, corresponding to a molar yield of 7%.

amend

Patent Claims

1. Process for preparing trifluoro-3(R)-hydroxybutyric acid derivatives of the generic formula



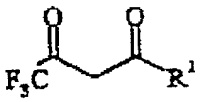
I

wherein

R^1 $-OR^2$, wherein R^2 is hydrogen, C_{1-10} alkyl, C_{1-10} alkenyl, C_{3-8} cycloalkyl, aryl, alkoxyalkyl or alkoxyalkoxyalkyl,

- NR^3R^4, wherein R^3 and R^4 are identical or different and are hydrogen, C_{1-10} alkyl, C_{1-10} alkenyl, C_{3-8} cycloalkyl or aryl, or
- SR^5, wherein R^5 is hydrogen, C_{1-10} alkyl, C_{1-10} alkenyl, aryl or C_{3-8} cycloalkyl,

comprising the conversion of a trifluoroacetoacetic acid derivative of the generic formula



II

wherein R^1 has the cited meaning, by means of microorganisms of the Escherichia species that are transformed with a gene coded for an enzyme which is capable of reducing a carbonyl function, or by means of a cell-free enzyme extract of this microorganism.

2. Process according to claim 1, characterized in that the biotransformation is conducted by means of microorganisms of the Escherichia coli JM109 species, Escherichia coli HB101 or Escherichia coli DH5.

3. Process according to one of claims 1 or 2, characterized in that the biotransformation is conducted by means of microorganisms of the Escherichia coli JM109 species, Escherichia coli HB101 or Escherichia coli DH5, which are transformed with genes that are coded for an enzyme, which is capable of reducing a carbonyl function, as well as for a glucose dehydrogenase.

4. Process according to claim 3, characterized in that the biotransformation is conducted by means of microorganisms of the Escherichia coli JM109 species or the Escherichia coli DH5 species, which are transformed with the plasmids pKAR and pKKGDH, as filed under the filing numbers DSM 11902 or DSM 12566.

5. Process according to one of claims 1 to 4, characterized in that the biotransformation is conducted at a temperature between 5 and 60° C.

6. Process according to one of claims 1 to 5, characterized in that the biotransformation is conducted at a pH between 5 and 10.

COMBINED DECLARATION AND POWER OF ATTORNEY

(Original, Design, National Stage of PCT, Divisional, Continuation or C-I-P Application)

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR PRODUCING TRIFLUORO-3(R)-HYDROXYBUTYRIC ACID DERIVATIVES

This declaration is of the following type:

[] original
[] design
[X] national stage of PCT.
[] divisional
[] continuation
[] continuation-in-part (C-I-P)

the specification of which: *(complete (a), (b), or (c))*

(a) [ ] is attached hereto.
(b) [ ] was filed on as Application Serial No. and was amended on *(if applicable).*
(c) [X] was described and claimed in PCT International Application No. PCT/EP99/01017 filed on February 18, 1999 and was amended on *(if applicable).*

Acknowledgement of Review of Papers and Duty of Candor

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of the subject matter claimed in this application in accordance with Title 37, Code of Federal Regulations § 1.56.

[ ] In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.98.

Priority Claim

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT International Application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT International Application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application on which priority is claimed

(complete (d) or (e))

(d) [ ] no such applications have been filed.
(e) [X] such applications have been filed as follows:

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION			
COUNTRY APPLICATION NO	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
Switzerland 0388/98	February 18, 1998		[X] YES NO [ ]
			[ ] YES NO [ ]
			[ ] YES NO [ ]
ALL FOREIGN APPLICATION[S], IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION			
			[ ] YES NO [ ]
			[ ] YES NO [ ]
			[ ] YES NO [ ]

Claim for Benefit of Prior U.S. Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Provisional Application Number	Filing Date

Claim for Benefit of Earlier U.S./PCT Application(s) under 35 U.S.C. 120

(complete this part only if this is a divisional, continuation or C-I-P application)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
PCT/EP99/01017	February 18, 1999	Pending

Power of Attorney

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge

that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE or COUNTRY	ZIP CODE
DATE	SIGNATURE OF INVENTOR			

Check proper box(es) for any added page(s) forming a part of this declaration

[ ] Signature for ninth and subsequent joint inventors. Number of pages added __________.

[ ] Signature by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor.
Number of pages added ______________________________.

[ ] Signature for inventor who refuses to sign, or cannot be reached, by person authorized under 37 CFR 1.47.
Number of pages added ____________________